PB-246 062

DEVELOPMENT OF A BIOLOGICAL MONITORING NETWORK - A TEST CASE - SUITABILITY OF LIVESTOCK AND WILDLIFE AS BIOLOGICAL MONITORS FOR ORGANOPHOSPHORUS CONTAMINANTS

W. W. Sutton, et al

Dugway Proving Ground

Prepared for:

National Environmental Research Center

June 1975

DISTRIBUTED BY:

NTIS

National Technical Information Service
U. S. DEPARTMENT OF COMMERCE

290013

EPA-680/4-75-003
JUNE 1975

PB 246 062

Environmental Monitoring Series

DEVELOPMENT OF A BIOLOGICAL MONITORING NETWORK -A TEST CASE

Reproduced by
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S Department of Commerce
Springfield, VA. 22151

NATIONAL ENVIRONMENTAL RESEARCH CENTER
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
LAS VEGAS, NEVADA 89114

RESEARCH REPORTING SERIES

Research reports of the Office of Research and Development, U. S. Environmental Protection Agency, have been grouped into five series. These five broad categories were established to facilitate further development and application of environmental technology. Elimination of traditional grouping was consciously planned to foster technology transfer and a maximum interface in related fields. The five series are:

1. Environmental Health Effects Research
2. Environmental Protection Technology
3. Ecological Research
4. Environmental Monitoring
5. Socioeconomic Environmental Studies

This report has been assigned to the ENVIRONMENTAL MONITORING series. This series describes research conducted to develop new or improved methods and instrumentation for the identification and quantification of environmental pollutants at the lowest conceivable significant concentrations. It also includes studies to determine the ambient concentrations of pollutants in the environment and/or the variance of pollutants as a function of time or meteorological factors.

EPA REVIEW NOTICE

This report has been reviewed by the National Environmental Research Center-Las Vegas, EPA, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the U.S. Environmental Protection Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation of use.

Document is available to the public for sale through the National Technical Information Service, Springfield, Virginia 22161.

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO. EPA-680/4-75-003	3. RECIPIENT'S ACCESSION NO. PB 246 062
4. TITLE AND SUBTITLE DEVELOPMENT OF A BIOLOGICAL MONITORING NETWORK - A Test Case - Suitability of Livestock and Wildlife as Biological Monitors for Organophosphorus Contaminants	5. REPORT DATE June 1975 6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) W.W. Sutton and L.L. Salomon	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Monitoring Systems Research and Development Laboratory National Environmental Research Center P.O. Box 15027 Las Vegas, NV 89114	10. PROGRAM ELEMENT NO. F11082 11. CONTRACT/GRANT NO. ROAP 22 ACS
12. SPONSORING AGENCY NAME AND ADDRESS Office of Research and Development U.S. Environmental Protection Agency Washington, D.C. 20460	13. TYPE OF REPORT AND PERIOD COVERED FINAL 14. SPONSORING AGENCY CODE

15. SUPPLEMENTARY NOTES

16. ABSTRACT

Upon request by the NERC-LV, a review was conducted of a DPG monitoring network which is designed to establish baseline erythrocyte acetylcholinesterase (AChE) levels in the fauna of West Central Utah, and to evaluate the suitability of using livestock and wildlife as biological monitors for organophosphorus contaminants. Wildlife species sampled during these DPG efforts included the antelope ground squirrel, the ORD Kangaroo rat, the deer mouse, and the black-tailed jackrabbit. Individual blood samples from these wildlife species as well as samples from cattle and sheep were collected and analyzed for red cell AChE activity. The analytical method employed was based on the Warburg manometric technique. Results indicate that the range of red cell AChE activity values for both livestock and wildlife species is sufficiently compact to allow observation of the depression of enzymic activity that would result from organophosphorus exposures. Controlled studies have shown that, following exposure to organophosphorus chemicals, the red cell activity recovers in an essentially linear fashion. Additive effects resulting from the simultaneous exposure to military agent VX and either toxic plants or commercial pesticides are discussed.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Monitoring Biological Monitors, Wildlife, Livestock Organophosphorus	Biological Monitoring Monitoring Network Animals as Monitors	0601 0603 0606 0616 0703 1400

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS *(This Report)* UNCLASSIFIED	21. NO. OF PAGES ~~52~~ 50
	20. SECURITY CLASS *(This page)* UNCLASSIFIED	22. PRICE 3.75

EPA Form 2220-1 (9-73)

GPO 693-743/51

EPA-680/4-75-003
June 1975

DEVELOPMENT OF A BIOLOGICAL MONITORING NETWORK

-A TEST CASE-

Suitability of Livestock and Wildlife As Biological Monitors for Organophosphorus Contaminants

By

W. W. Sutton
Monitoring Systems Research and Development Laboratory
National Environmental Research Center
P. O. Box 15027
Las Vegas, NV 89114

L. L. Salomon
Test Operations Directorate
Dugway Proving Ground
Dugway, UT 84022

Contract No. B10019
ROAP 22 ACS
Program Element No. 1HA325

1/

Prepared for
OFFICE OF RESEARCH AND MONITORING
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

ABSTRACT

Upon request by the National Environmental Research Center-Las Vegas, a review was conducted of a Dugway Proving Ground (DPG) monitoring network which is designed to establish baseline erythrocyte acetylcholinesterase (AChE) levels in the fauna of West Central Utah, and to evaluate the suitability of using livestock and wildlife as biological monitors for organophosphorus contaminants.

Wildlife species sampled during these DPG efforts included the antelope ground squirrel (*Ammospermophilus leucurus*), the Ord kangaroo rat (*Dipodomys ordii*), the deer mouse (*Peromyscus maniculatus*), and the black-tailed jackrabbit (*Lepus californicus*). Individual blood samples from these wildlife species as well as samples from cattle and sheep were collected and analyzed for red cell AChE activity. The analytical method employed was based on the Warburg manometric technique.

Results indicate that the range of red cell AChE activity values for both livestock and wildlife species is sufficiently compact to allow observation of the depression of enzymic activity that would result from organophosphorus exposures. Controlled studies have shown that, following exposure to organophosphorus chemicals, the red cell activity recovers in an essentially linear fashion. Additive effects resulting from the simultaneous exposure to military agent VX and either toxic plants or commercial pesticides are discussed.

CONTENTS

	Page
Abstract	ii
List of Figures	v
List of Tables	vi
Acknowledgments	vii
Conclusions	viii
Recommendations	ix
Background	1
The Pesticide Problem	1
Application of Biological Monitors	1
Overview of Some Supporting DPG Programs	1
Ecological Surveys	2
Toxicology Studies	3
Transport Processes	3
Soil, Vegetation and Water	3
Meteorological Transport	3
Instrument Evaluation	4
Modeling Capability	5
EPA Interest in the DPG Organophosphorus Monitoring Programs	6
Monitoring Methods and Materials	7
Field Techniques	7
Analytical Techniques	10
Results and Discussion	14
Field Survey	14

CONTENTS cont'd

	Page
Control Studies in Support of Field Program	19
Acetylcholinesterase Determinations Using Hereford Steers	19
Toxicity of VX in Hereford Steers and Sheep	23
Cattle as Indicator Animals	25
Acetylcholinesterase Determinations Using Range Sheep	25
Results from Studies Using Laboratory Animals	25
Studies on Wildlife Species (Small Mammals)	26
Studies on Wildlife Species (Fish)	26
Complementary Toxicological Effects of Plant Poisons and Chemical Agents on Mammals	27
Evaluation of Complementary Effects Between Military Chemical Agents and Organophosphorus Pesticides	30
References Cited	32
Appendix	33

LIST OF FIGURES

FIGURE		PAGE
1.	Schematic representation of a typical trap line for wildlife collections	8
2.	Diagrammatic erythrocyte acetylcholinesterase activity recovery pattern following various degrees of inhibition	12
3.	Mean dose response curve showing the relationship between oral VX exposures of 0.1 and 0.3 µg/kg/day for 56 days and the level of erythrocyte acetylcholinesterase activity. Four Hereford steers were used in each treatment group.	20
4.	Mean dose response curve, depression and recovery phases in Hereford steers showing the relationship between oral VX exposures of 0.1, 0.2, and 0.3 µg/kg/day for 56 days plus an exposure of 0.7 µg/kg/day for 75 days and the level of erythrocyte acetylcholinesterase activity	21
5.	Mean dose response curve, depression and recovery phases, in three Hereford steers showing the relationship between oral VX doses of 1.0 µg/kg/day for 75 days and the level of erythrocyte acetylcholinesterase activity	22
6.	Mean dose response curve, depression and recovery phases, in three Hereford steers showing the relationship between oral VX doses of 2.2 µg/kg/day for 75 days and the level of erythrocyte acetylcholinesterase activity	22

Preceding page blank

LIST OF TABLES

TABLE		PAGE
1.	Abbreviated Habitat Classification System Used In Categorizing Various Wildlife Collections	9
2.	Erythrocyte Acetylcholinesterase Activity in Four Species of Small Mammals Indigenous to the Bonneville Basin of West Central Utah - Spring Collections 1973	15
3.	Erythrocyte Acetylcholinesterase Activity in Four Species of Small Mammals Indigenous to the Bonneville Basin of West Central Utah - Fall Collections 1973	16
4.	Erythrocyte Acetylcholinesterase Activity Mean Values in Sheep and Cattle in Bonneville Basin of West Central Utah - Fall 1970	17
5.	Erythrocyte Acetylcholinesterase Activity Mean Values in Sheep and Cattle in Bonneville Basin of West Central Utah - Spring 1971	17
6.	Erythrocyte Acetylcholinesterase Activity Mean Values in Sheep and Cattle in Bonneville Basin of West Central Utah - Fall 1971	18
7.	Erythrocyte Acetylcholinesterase Activity Mean Values in Sheep and Cattle in Bonneville Basin of West Central Utah - Spring 1972	18
8.	Toxicity of VX in Sheep	24

Preceding page blank

ACKNOWLEDGMENTS

This report was prepared through EPA Contract No. B10019 to Dugway Proving Ground. Selected experimental data from field and laboratory studies, previously collected by DPG scientists, were reviewed and discussed in terms of the EPA stated interest in biological monitoring. Portions of the document were taken from DPG reports and additional material was provided specifically for EPA. The document was compiled and written by W. W. Sutton and L. L. Salomon.

CONCLUSIONS

1. The dispersion of baseline erythrocyte AChE activity levels in cattle, sheep, black-tailed jackrabbits, deer mice, Ord kangaroo rats and antelope ground squirrels is sufficiently small, relative to mean values, to allow detection of enzymic depressions resulting from organophosphorus exposure.

2. The method employed to analyze for red cell AChE, a modified Warburg manometric technique, has provided reproducible, accurate, and timely information. Furthermore, this method does not require highly skilled personnel or expensive equipment. Other relatively simple methods of AChE analysis are available which may prove equally valid or more economical for use at other laboratories.

3. Following organophosphorus exposure, red cell activity recovers in an essentially linear manner that is dependent on erythropoiesis and the life of the individual red cells. If, for example, red cell AChE depression is noted in a biannual survey of range cattle, two or three subsequent collections taken at twenty-day intervals would establish the slope of the recovery curve. By extrapolation, the original degree of enzymic depression and the last date of pesticide exposure could be estimated.

4. Analysis of red cell AChE activity is most applicable as a screening technique since it does not permit identification of the individual organophosphorus substance responsible for enzymic depression. AChE assays do provide a measure of actual effect on the biological material as they serve to detect the highly specific biochemical reaction common to all organophosphorus exposures. Once enzymic depression is established and the ranchers, wildlife managers, agricultural officials, etc., questioned, a series of more involved and expensive procedures can be initiated to determine the specific cholinesterase inhibitor, provided interrogation does not resolve the problem.

RECOMMENDATIONS

Analogous studies to calibrate the red cell AChE response in terms of commercial pesticide units should be conducted in various indicator species, and field programs should be initiated in areas where intensive agricultural use is made of organophosphorus pesticides. Results from such a combined field and laboratory effort could be compared with the DPG survey results, as the DPG perimeter survey provides data from a control zone known to be essentially free of organophosphorus pollutants.

BACKGROUND

THE PESTICIDE PROBLEM

The continually expanding human population requires an abundant food supply. This requirement is being achieved partially through the introduction of high-yield crop varieties, improved irrigation, new crop management techniques such as multiple cropping, as well as more effective farm machinery, fertilizers and, of course, pesticides. Pesticides present a somewhat unique situation as far as environmental protection is concerned since these particular toxic substances are intentionally released into the environment with a desired lethal effect, albeit for a restricted group of organisms. The current concern over DDT and other persistent halogenated hydrocarbons has resulted in an expanding use of the generally less persistent organophosphorus and carbamate insecticides and, therefore, a growing need for monitoring to avert or detect adverse environmental effects from such compounds.

APPLICATION OF BIOLOGICAL MONITORS

Historically, most monitoring efforts have assessed a single pathway system only, even when total pollutant exposure was the ultimate goal. To establish the total potential exposure from air, water, soil, and food material, monitoring programs are needed that provide an integrating function. Biological monitors appear to be uniquely suited since, as in the case of many terrestrial mammals, atypical biotic characteristics can often be detected following various modes of pollutant exposure, i.e., oral (food or water), inhalation and percutaneous. Through inhibition or alteration of metabolic processes, as well as retention of many pollutant substances by the tissues, biological organisms can be employed in many monitoring situations.

OVERVIEW OF SOME SUPPORTING DPG PROGRAMS

Dugway Proving Ground which was activated in 1951 as a permanent military installation for the testing of chemical and biological weapons systems developed monitoring programs as a logical operational requirement. An integrated monitoring effort was initiated to detect any adverse ecological effects that might result from the testing program. This monitoring effort included a broad spectrum of detailed ecological, chemical, meteorological, epidemiological and toxicological studies to provide sound environmental baseline data for West Central Utah, an area approximately 15,000 square miles in size.

It was recognized that there is no universally applicable formula for an integrated monitoring program. The ecological characteristics of the specific area or region, the type(s) and source(s) of pollutants, the nature of the specific objectives to be met, and considerations of resources and time, all enter into the selection of activitives to achieve practically and scientifically acceptable goals.

Ecological Surveys

For its purposes, DPG considered establishment of ecological baseline data to be a key factor in an integrated monitoring program to determine whether military activities impact adversely upon the local environment, and to define ecological receptors and transport pathways that may be critical in the event of pollutant release. The preliminary hypothesis was that no significant adverse effects on flora or fauna resulted from the activities, e.g., the employment of organophosphorus compounds, and this hypothesis was tested by evaluation of comprehensive empirical data.

Information collected on wildlife species includes population density and diversity, seasonal and migration cycles, predator-prey relationships, intra or interspecific competition for food, grazing competition from domestic livestock, depredation by parasites and disease, plus any additional related effects resulting from variations in rainfall, temperature, soil type and soil fertility. It was also important, in certain cases, to determine those factors which influence the host-parasite relationship within the ecosystem of interest and which allow for the maintenance of infection and foster or inhibit epizootics. Studies or literature reviews are also undertaken at DPG to analyze wildlife feeding habits to assess the potential effects of agricultural or military pollutants upon the animal feeding patterns, upon the foods themselves and upon adverse reactions occurring under natural conditions. Furthermore, an analysis and classification of various soils according to type, particle size, water permeability, water content, acidity-alkalinity and the amounts and types of mineral salts are also a part of the baseline study. Seasonal analyses are needed to determine the locations and amounts of surface water and to define the conditions that effect the subsurface water and its movements and/or drainage. Plant surveys provide data for evaluating the effects of plants upon wildlife and/or domestic animals (livestock) and for assessing the effects of poisonous plants in combination with toxic chemical agents. This program of ecological monitoring and surveillance is being achieved, within the framework of available resources and time, by careful selection of control zones where all major factors of ecological consequence are equally and simultaneously operative except that there is no possibility of influences attributable to military operations. In short, the need for minutely detailed investigations is being obviated by establishing appropriate control zones distant to the test zones (sites selected because of their sensitive location relative to military testing). Significant ecological divergences between control and test zones, particularly differences in trends, may then be used to reevaluate the selection of control sites and/or the occurrence of unfavorable events traceable to military operations.

Toxicology Studies

A number of toxicology studies were undertaken as an adjunct to the ecological monitoring network. Toxicity levels have been established for various agents and decomposition products using laboratory and indigenous small mammals. In addition, systematic acute and chronic toxicity feeding studies have been conducted using large domestic animals of economic importance. These studies were primarily conducted to determine the AChE depression effected by administration of lethal agents. Information gained from these projects was applied to studies in which animals were used as indicators of residual toxic materials present in vegetation. Possible synergistic effects between naturally occurring plant materials and agents were also investigated.

Transport Processes

As was the case with the other studies on organophosphorus military agents, these DPG investigations on transport pathways were designed to be comparable with techniques and methods commonly employed to measure pesticides. In several instances, pesticides were actually used to verify analytical procedures and in persistence studies a rather extensive data bank related to pesticides, herbicides, etc., has been developed. It was found that while much information was available on halogenated pesticides, only fragmentary information as to methodology and environmental fate was available for many of the organophosphorus pesticides.

Soil, vegetation and water

The fate of organophosphorus chemical agents has been examined in soil and vegetation under carefully controlled conditions simulating various aspects of the Dugway environment. As a result, the hydrolysis routes were defined and the hydrolysis products of the neuro-toxic agents were identified. The major mechanisms by which these chemicals degrade have been established. Investigations into the uptake, translocation and metabolic fate of these chemicals by plants have also been completed.

Detection and measurement of traces of lethal agents and organophosphorus pesticides in water have also been conducted; as little as 0.4 parts per billion can be detected, a level far below that which is toxic to living matter. Furthermore, even though these materials decompose in water, the decomposition products were quantitatively accounted for up to one year after deposition.

Meteorological transport

Pesticides are generally introduced into the ecosystem through some type of spraying (aerial or surface) operation, therefore, the air route is especially important. Both the efficiency of the pesticide

application and the pesticide dispersal to non-target areas are influenced by local meteorological conditions. Meteorogological transport characteristics are also extremely important for the analysis of military chemicals. To be effective, a military chemical agent much like an agricultural poison, must be properly disseminated over a suitable target area. Dissemination of a military chemical comprises projection, or delivering the chemical to the target, and dispersion or spreading of the agent over the target in an effective manner. Environmental studies involving organophosphorus chemicals at DPG were, therefore, intimately concerned with the transport and diffusion of airborne vapors and droplets. Knowledge of the droplet diameter, fall velocity and evaporation rate as well as the volume fraction of the aerosol which has a high probability of being subjected to drift have improved the prediction capability of long range contamination. The size of the droplets directly relates to their ability to impact on the target and if the droplet size is too small the tendency of the aerosol to drift is increased. Meteorology programs for the study of transport of agent clouds were established to (1) evaluate and describe the influence of complex environmental features such as terrain and vegetation on the processes of aerosol transport and diffusion, (2) to select optimum monitoring techniques and (3) to assess the efficiency of various chemicals and release mechanisms. During the past decade, almost five hundred major field diffusion experiments were conducted through a combination of inhouse (DPG) efforts and contractor support. Emphasis was placed on mesoscale studies (1 to 100 kilometers) and included topographic variations consisting of a single or a multiple valley complex, a single open airshed or a single geographic feature. Open air releases of organophosphorus aerosols, gases and simulant materials have provided valuable data for field assessment of various air monitoring networks. These studies were based on (1) the theoretical development of mathematical models for atmospheric transport and (2) validation of the theoretical models with data derived from field experimentation. Early in the process of generating mathematical models and practical operational information, it was surmised that vast amounts of the necessary information could be acquired by using a harmless tracer, e.g., fluorescent particles, whose behavior upon dispersal in the atmosphere is identical to that of clouds of agents. The size and number of spray drops impinging on the target can be determined since the number of fluorescent particles in each droplet is a direct measure of droplet size.

Additional field experiments are being conducted to determine the quantity of toxic material that is resuspended by wind action. Prior to experiments of this sort, extraction methods and recovery techniques were developed. The decomposition and decay of agents has received particular attention so that determinations could quantify the amount of agent available for resuspension.

Instrument Evaluation

Since monitoring networks require high sensitivity samplers, a continuous program of instrument evaluation is in existence, with

particular emphasis on the detection of organophosphorus aerosols and gases. In recent studies a flame photometric detector (FPD) was evaluated for use as a field monitor of agent concentration. Analytical units, such as FPD, are employed in the field during selected tests or in specifically designed chamber tests. Data from field trials have been used to assess instrument desirability and to establish engineering and design changes for the next generation prototype.

In support of air tracer studies for aerosol or particulate transport several surface point sources and aircraft disseminator systems are being employed together with rotorod (impactor) samplers, millipore filters, and aircraft sampling devices. Assessment of tracer samples is routinely accomplished by microscopic techniques with portable assessment systems; an automated system is now being constructed. Turbulence statistics measurement programs required to apply diffusion theories to practical studies of aerosol or air pollutant movement can be aided by two instrument packages (for aircraft) consisting of the following components:

(a) Doppler radar (measurements of Vertical Wind Profile)
(b) Temperature probe (ambient air)
(c) Relative humidity probe
(d) Infrared ground surface temperature probe
(e) Air turbulence indicator
(f) Air speed and altitude indicator
(g) Multi-channel strip recorder

This system was used extensively in the past on safari operations in areas where other methods of data acquisition were either impossible or impractical.

Modeling Capability

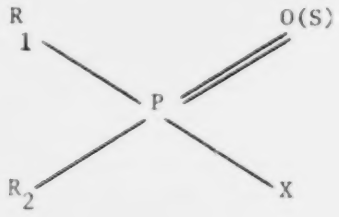
A complete modeling capability has been established at DPG with particular emphasis on mathematical descriptions for the diffusion and transport of airborne substances. These models, adapted for computer solution, have been validated utilizing data from the weapons test program and the above mentioned fluorescent particle tracer technique.

Modeling developments, which have been incorporated into capabilities for the field Army, have also been utilized in hazard evaluation for specific Department of Defense problems. Hazard prediction techniques were developed in case of an accidental release during the transportation of organophosphorus chemicals by truck, rail and/or ship. Area source models have also been applied to the evaluation of downwind concentration levels for planned Herbicide Orange disposal operations.

EPA INTEREST IN THE DPG ORGANOPHOSPHORUS MONITORING PROGRAMS

Generally, organophosphorus pesticides are similar to organophosphorus military agents in chemical structure, biological activity, or both. As a result, it is felt that these detailed environmental investigations may serve as prototypes for corresponding efforts on the organophosphorus pesticides.

The relationship between these pesticides and the military agents is seen in the general formula for cholinesterase inhibitors of the organophosphorus group.



"R_1 and R_2 are capable of almost infinite variation. They may represent alcohols, phenols, mercaptans, amides, or alkyl or aryl groups attached directly to the phosphorus, etc. Common X radicals are from fluorine (e.g., in diisopropylfluorophosphate) paranitrophenol (e.g., in Paraoxon), and phosphates (in a pyrophosphate, tetraethylpyrophosphate), but in other inhibitors X may be cyanide, thiocyanate, carboxylate, chloride or almost any phenoxy or thiophenoxy group" (Holmstead, 1963). Generally, organophosphorus compounds containing a double-bonded sulfur atom are low in pesticide activity, toxicity, and AChE inhibitory activity. Upon metabolic conversion or rearrangement to the corresponding oxygen derivative, the toxicity tends to rise dramatically along with their effectiveness as an inhibitor of AChE.

Military nerve agents are selected from this general group to meet necessary biological, physical, and chemical properties including stability and toxicity by a given route of entry into the body. Similarly, organophosphorus pesticide selection is based on a number of criteria related to their specific use. Some organophosphorus pesticides have LD_{50} values close to those of nerve agents, but they are usually less volatile. An amount of nerve agent giving a certain degree of biological effect is not intrinsically more hazardous or toxic than an amount of organophosphorus pesticide giving the same degree of biological effect.

MONITORING METHODS AND MATERIALS

FIELD TECHNIQUES

Originally the monitoring program utilized such indigenous small mammals as the black-tailed jackrabbit, Townsend ground squirrel, antelope ground squirrel, least chipmunk, Great Basin pocket mouse, Ord kangaroo rat, chisel-toothed kangaroo rat, bushy-tailed wood rat, grasshopper mouse, long-tailed pocket mouse, desert cottontail, western harvest mouse, deer mouse, pinyon mouse and desert wood rat. However, the wildlife monitoring effort for the detection of anti-cholinesterase substances was condensed to include four sentinel or indicator species. These were the antelope ground squirrel *(Ammospermophilus leucurus)*, the Ord kangaroo rat *(Dipodomys ordii)*, the deer mouse *(Peromyscus maniculatus)* and the black-tailed jack-rabbit *(Lepus californicus)*. Selection of the species was based on their wide distribution, availability in terms of numbers and season, and ease of sampling. The antelope ground squirrel is active through-out the year and inhabits the vegetated and semi-vegetated dunes of the valleys and the sandier parts of the foothills. *Peromyscus maniculatus*, the ubiquitous deer mouse, is the most cosmopolitan and numerous rodent in the area. It is especially abundant in the vegetated dunes along the valley floors and in the sandy areas of the foothills. The Ord kangaroo rat is most prevalent in the vegetated dunes and among mixed brush in the lower foothills. Finally, the black-tailed jack-rabbit occurs in essentially all plant communities of the valleys, foothills and mountains, but the populations fluctuate from year to year.

Seven collection sites were chosen to include locations of vary-ing distance from the Proving Ground, so that all directions surround-ing the test grids would be represented and a variety of microhabitats would be included. Particular consideration was given to the prevailing wind conditions for the area. Since seasonal population movements often occur, especially among jackrabbits, each survey site represented a fairly large area to insure collection of an adequate number of mammals throughout the year. Can traps, consisting of one-quart cans with museum special snap traps, were used for rodent collections. Traps were baited with a mixture of seeds and were checked daily in the early morning. During the winter months, cotton batting material was placed in the traps to avoid exposure fatalities. Lines of 40 can traps, with an interval of 6 to 8 paces between traps, were set in an elliptical pattern (Figure 1) in areas which visual reconnaissance indicated would be productive. The most fruitful areas were found to be vegetated sand dunes on which adequate cover and numerous burrows were observed. An abbreviated classification system (Table 1) for the various habitat types proved useful in categorizing the collection. Jackrabbits were collected from each sentinel area using firearms. Since night collect-ing was usually more productive in areas of reduced animal density,

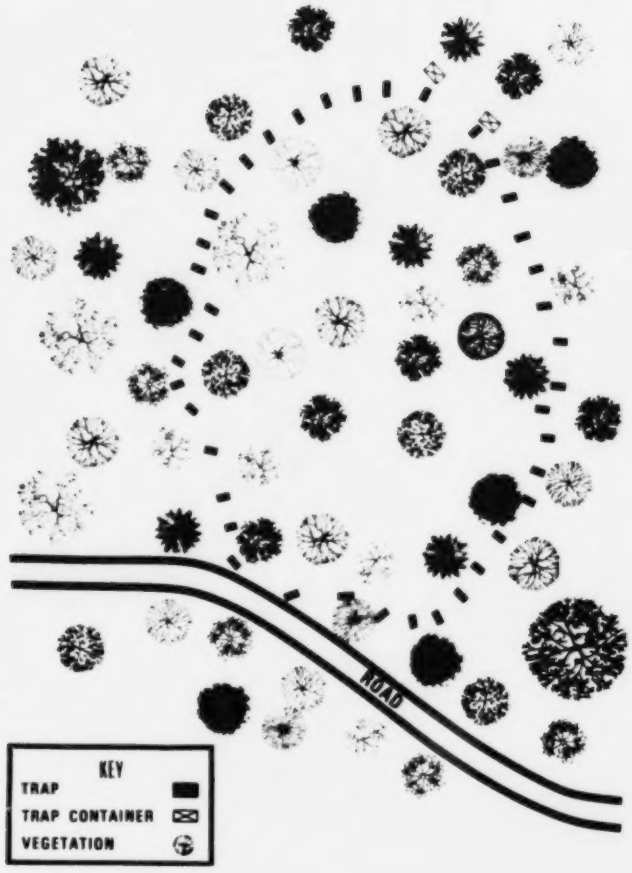


FIGURE 1. Schematic representation of a typical trap line for wildlife collections

hunting was conducted from the back of a truck equipped with spotlights. If an area had a large rabbit population the hunting was done on foot during daylight hours. All blood samples were taken in the field by cardiac puncture using 10-ml heparinized Vacutainers and 1.5-inch, 20-gauge needles. After the blood was collected, the Vacutainers were immediately placed in a portable ice chest and transported back to the laboratory for processing. Rodents, which had been live-trapped, were placed in small cloth bags and returned to the laboratory for bleeding.

Table 1. ABBREVIATED HABITAT CLASSIFICATION SYSTEM USED IN CATEGORIZING VARIOUS WILDLIFE COLLECTIONS

Biotic Community	Code Recorded on Individual Mammal Collection
Pond	1
Marsh	2
Stream	3
Irrigated Area	4
Cultivated Area	5
Greasewood	6
Juniper Brush	7
Juniper Mountain	8
Pinyon - Juniper	9
Mixed Brush	10
Shadscale-Graymolly	11
Shadscale-Graymolly-Greasewood	12
Vegetated Dunes	13
Rabbitbrush	14
Big Sage	15
Big Sage - Rabbitbrush	16
Spring	17
Natural Cave	18
Mine	19
River	20
Lake	21
Grass-Annuals	22
Greasewood - Sagebrush	23
Rabbitbrush - Greasewood	24
Rocky Hillsides Sparse Vegetation	25
Aspen - Fir	26
Shadscale - Sagebrush	27

If the distance to the laboratory was such that this was impractical, field collections were taken by cardiac puncture using 0.5 ml heparinized tuberculin syringes with 3/8-inch, 26-gauge needles. The blood was then transferred to 3-ml heparinized tubes and, as in the case of rabbit samples, refrigerated until it could be processed. Collections of both rodents and lagomorphs were catalogued by species, sex, location, habitat, and date collected.

Wildlife controls were maintained at the DPG Faunal Colony. The following species were sampled in conjunction with the perimeter survey.

Jackrabbits (*Lepus californicus*)
Wood rats (*Neotoma lepida*)
Kangaroo rats (*Dipodomys sp.*)
Grasshopper mice (*Onychomys leucogaster*)
Harvest mice (*Reithrodontomys megalotis*)
Canyon mice (*Peromyscus boinitus*)
Pinyon mice (*Peromyscus truei*)
Deer mice (*Peromyscus maniculatus*)

As was the case with wildlife collections, livestock samples were teken from several locations of varying distance from the Proving Ground with particular attention being given to prevailing wind conditions. In addition to these range animals sampled on the DPG perimeter, blood collections were taken from sheep and cattle at the DPG Animal Colony. Lambs, pregnant and nonpregnant ewes, wethers and mature Hereford steers were kept under controlled conditons to quantitate AChE activity variations. Lambs were bled weekly for six months to determine if any change in red cell activity occurred prior to maturity. The DPG Animal Colony sheep and cattle were, of course, not exposed to military or agricultural cholinesterase inhibiting agents. Activities recorded in the spring and fall were of special importance since the field collections were made at this time.

Perimeter and DPG control livestock blood samples were taken from the jugular vein using 10-ml 100 X 16 mm heparinized vacutainers with a 20-gauge X 1-1/2" needle. Samples were cooled and transported to the laboratory for analysis.

ANALYTICAL TECHNIQUES

Exposure to low levels of organophosphorus pesticides does not characteristically result in detectable tissue accumulation. However, since these compounds are degraded metabolically and are excreted in the urine, determination of the urinary metabolites, e.g., phenols, phenoxy acids and alkyl phosphates, can be used as a means of assessing exposure and of establishing with more or less specificity the identity of the pollutant. A property common to all organophosphorus pesticides is their inhibitory effect on AChE. This, too, can serve as the basis of detecting exposure, but not of identification. It remained, therefore,

to select that analytical method which best met the requirements of the monitoring effort.

The physiological effects resulting from the systemic absorption of organophosphates and carbamates are caused by inhibition of cholinesterase enzymes of the nervous system, muscles and secretory glands. Phylogenetically, the earliest function of acetylcholine (ACh) and related enzymes was probably to modify the passage of various substances across cell membranes. With the evolution of structural and biochemical complexity this function was retained by some membranes and totally lost by others. The greatest specialization has been achieved in the nervous tissue where the ACh-AChE system, by virtue of its effect on the ionic permeability of membranes, functions as a transjunctional mediator. In the case of erythrocyte AChE, the enzyme appears to be vestigial but it may too affect ionic permeability in some nonessential way. Cholinesterase enzymes of plasma and red blood cells are also inhibited by organophosphorus and carbamate chemicals but this has no apparent effect on the animal's health. Acetylcholinesterase may be inhibited in a reversible or an irreversible manner. While these are not absolute distinctions, reversible inhibition generally indicates that the enzyme-inhibitor complex dissociates freely upon removal of the inhibitor and irreversible inhibition implies that the enzyme activity does not readily return on mere dialysis. Organophosphorus compounds phosphorylate cholinesterase to form a dialkylphosphorylated enzyme which is an irreversible complex unlike the easily dissociated carbamate-enzyme complex. Recovery of activity from an irreversibly inhibited enzyme will depend on the rate of regeneration of new enzyme protein. In the case of red cells, new cells must be formed and recovery of total erythrocyte activity is dependent on the rate of erythropoiesis and the normal red cell life span.

Analytical methods for the detection and quantitation of excretion products derived from organophosphorus pesticides are generally demanding in time, skills, and equipment. By their very nature, these methods impose a limitation on the information obtainable from the monitoring effort unless multiple analyses are performed at correspondingly increased cost. Furthermore, usable concentrations of metabolic residues are found in the blood and urine for a limited time only after pesticide exposure and false positives may be recorded if the animals ingest relatively harmless organophosphorus degradation products. Urine is also difficult to sample from unconfined animals and effectively useless as a source of routine monitoring specimens. Measurements of urinary residues are also difficult to correlate with the actual hazard caused by the organophosphorus chemicals, which are more accurately reflected by the degree of red cell AChE inhibition.

Determination of erythrocyte AChE levels indicate the degree of actual damage to the biological material as they serve to detect the highly specific biochemical reaction common to all organophosphorus pesticide exposures. Admittedly, red cell AChE analysis represents a

screening method only since it does not permit identification of the individual organophosphorus pesticide(s) responsible for the enzymic depression, but the results are not complicated by prior ingestion of harmless pesticide degradation products. As the rate of enzymic recovery is predictable, it is possible to estimate the date of an acute exposure or the date of the last day of a chronic exposure. If recovery is not linear and/or of the proper slope, it suggests that the animals are still being exposed to the toxic materials. In biological monitoring projects designed for environmental surveillance, a series of field collections would indicate the slope of the recovery curve if the animals had previously been exposed to an anticholinesterase substance. If, for example, red cell AChE depression is noted in a biannual survey of range cattle, two or three subsequent collections taken at twenty-day intervals would establish the slope of the recovery curve. By extrapolation, the original degree of enzymic depression and the last date of pesticide exposure could be estimated. Figure 2 presents a diagrammatic view of erythrocyte AChE recovery which could be used to classify the slope of the recovery curve. The figure could also be viewed as establishing three arbitrarily selected zones. Activity increases corresponding to Zone I would indicate a need for increased surveillance of a given area, but conclusions concerning the degree of contamination should be reached cautiously since

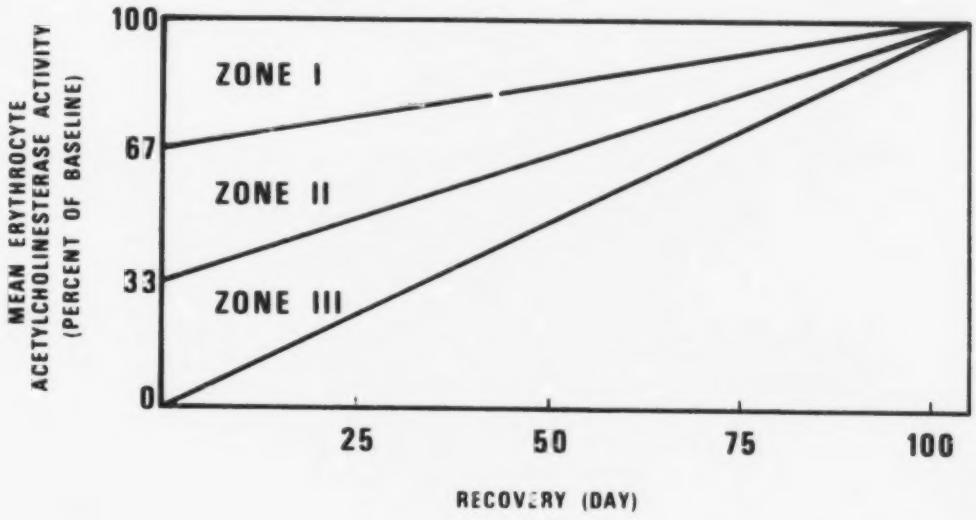


FIGURE 2. Diagrammatic erythrocyte acetylcholinesterase activity recovery pattern following various degrees of inhibition

normal AChE activity levels fall within a fairly broad range. An apparent AChE recovery falling within Zone II would indicate probable exposure to a cholinesterase inhibitor while a recovery sequence with Zone III boundaries would suggest the presence of a significant degree of contamination. Theoretically, it would be possible to mathematically model the red cell enzymic recovery rate following various types of organophosphorus exposures. However, for practical field monitoring purposes, the recovery pattern that would essentially fall within one of these three arbitrarily selected zones can provide an extrapolated approximation of the last exposure date.

For these reasons, and since organophosphorus chemicals cause no other major effect than that attributable to cholinesterase inhibition, measurement of AChE activity was judged to be the only realistic and practical approach to monitor for the effectiveness of the ecological protection program of DPG. An ancillary benefit is also realized from the wildlife collection part of the screening program because population density, age distribution, and species composition can reflect many nonrelated detrimental effects of human operations. Capabilities for more detailed chemical and pathological investigations were also established, but with no plans for routine employment of that support unless required by findings made during the screening effort.

Witter (1963) and Augustinsson (1954) have reviewed several of the acetylcholinesterase assay techniques. These reviews included discussions of methods based on acid production, choline production, chemical determination of unreacted acetylcholine, and use of non-choline esters. Additional papers have discussed radioisotopic techniques suitable for AChE assays (Reed *et al.*, 1966 and Gaballah, 1968).

A gasometric method of analysis for red cell AChE, employing the Warburg manometric technique* was selected for the DPG monitoring program because it, in effect, is the ultimate reference standard. The procedure is described in Appendix I.

*For a more elaborate and fundamental discussion of the principles and classic manometric techniques utilizing the Warburg respirometer, the reader is referred to Umbreit, W. W., R. H. Burris, and J. F. Stauffer, 1949. Manometric Techniques and Tissue Metabolism. Burgess Publishing Co., Minneapolis, MN. 227 pp.

RESULTS AND DISCUSSION

A survey and surveillance program has been maintained to establish baseline levels of erythrocyte acetylcholinesterase (AChE) activity in representative wildlife and livestock species. This program has provided quantitative verification of the concept, solutions to logistical and analytical problems, development of an appropriate quality assurance program, information on fiscal and personnel requirements, and most importantly yielded sufficient data to establish the baseline information for the geographic area under investigation. The fact that these collection efforts were designed to establish baseline information must be stressed, not only because baseline surveys are an essential part of integrated monitoring but also because DPG did not test toxic organophosphorus materials in the open air for many months prior to, nor at any time after inception of this study. Furthermore, results of residue analysis of soil, water, and vegetation, concurrently covering the same area as the livestock and wildlife surveillance effort, did not show the presence of any organophosphorus compounds.

FIELD SURVEY

Tables 2 through 7 present representative AChE activity data from livestock and wildlife species collected in areas adjacent to DPG. The observed variations in erythrocyte activity levels in no way interfere with the reliability and consistency with which depressed values can be interpreted. Anderson *et al.* (1969) presented red cell activity values for large numbers of sheep and cattle and concluded that the range of values in these animals was sufficiently small so as to present no handicap in recognition of reductions in enzymic activity caused by organophosphorus poisoning. The standard deviation calculated for these (Anderson's) data revealed a dispersion of AChE activities about the mean startlingly similar to that noted in the DPG survey. Actually, the data obtained by DPG for AChE in erythrocytes of sheep are more closely clustered about the mean, Anderson's data having a number of outliers (which were disregarded in calculating the standard deviation).

To be useful as sentinels, wildlife species will need to be adequately distributed geographically, and available in terms of numbers, season and ease of sampling. Furthermore, as with the cattle and sheep, the baseline activity of AChE in erythrocytes should be sufficiently compact to allow accurate interpretation of changes owing to encounters with organophosphorus compounds. The wildlife data obtained by DPG showed, in most cases, a greater central tendency than did AChE values from domestic animals, although they were based on a smaller number of specimens. *Lepus californicus* and *Peromyscus maniculatus* are probably the most successful sentinel candidates both in regard to availability and dispersion of enzymic activity. Levels of activity in red cells of jackrabbits are normally low which might produce some difficulty in determining significant changes were it not for the fact that they

Table 2. ERYTHROCYTE ACETYLCHOLINESTERASE ACTIVITY* IN FOUR SPECIES** OF SMALL MAMMALS INDIGENOUS TO THE BONNEVILLE BASIN OF WEST CENTRAL UTAH - SPRING COLLECTIONS 1973

Area	Species	Sample Size	Activity Mean	Standard Deviation	95% Confidence Interval
Callao	D.o.	9	45.9	5.7	41.5-50.3
	P.m.	28	47.1	6.9	44.4-49.8
	L.c.	10	20.9	2.8	18.9-23.0
Cedar Mtns. South	D.o.	18	76.7	10.9	71.3-82.2
	P.m.	17	49.0	11.8	42.9-55.1
	L.c.	9	20.4	2.1	18.8-22.0
Condie	A.l.	1	27.7		
	D.o.	11	65.5	9.9	58.9-72.2
	P.m.	19	53.6	7.4	50.1-57.2
	L.c.	9	19.2	3.9	16.2-22.2
Fish Springs	A.l.	7	68.1	11.0	57.9-78.2
	D.o.	12	65.7	11.0	58.7-72.7
	P.m.	13	53.7	10.6	47.3-60.1
	L.c.	10	22.7	3.3	20.3-25.1
Gold Hill	P.m.	33	49.7	10.6	46.0-53.4
	L.c.	10	20.7	2.3	19.0-22.3
Government Creek	D.o.	23	61.0	6.8	58.1-63.9
	P.m.	15	43.7	9.7	38.3-49.0
Granite Mtn.	D.o.	26	59.1	18.4	51.7-66.5
	L.c.	10	21.5	4.3	18.4-24.6
Iosepa	P.m.	27	52.1	9.2	48.4-55.7
	L.c.	2	21.4	0.5	17.1-25.7
Wendover	A.l	2	82.4	3.8	48.0-116.7
	D.o.	5	59.7	7.7	50.1-69.3
	P.m.	6	43.4	9.1	33.9-52.9
	L.c.	5	20.7	3.4	16.4-24.9

*Activities for *Lepus californicus* are reported as µM CO_2 evolved per 100 µl RBC's per 30 minutes. Activities for all other species are reported as µM CO_2 evolved per 50 µl RBC's per 30 minutes.

**Species: A.l. - *Ammospermophilus leucurus*, D.o. - *Dipodomys ordii*
P.m. - *Peromyscus maniculatus*, L.c. - *Lepus californicus*

Table 3. ERYTHROCYTE ACETYLCHOLINESTERASE ACTIVITY* IN FOUR SPECIES** OF SMALL MAMMALS INDIGENOUS TO THE BONNEVILLE BASIN OF WEST CENTRAL UTAH - FALL COLLECTIONS 1973

Area	Species	Sample Size	Activity Mean	Standard Deviation	95% Confidence Interval
Callao	P.m.	30	43.2	11.8	38.8-47.7
	L.c.	5	20.9	3.5	16.5-25.1
Cedar Mtns. South	A.l.	2	50.6	10.5	43.7-145.0
	D.o.	20	73.4	16.0	66.0-80.9
	P.m.	8	44.7	21.4	26.8-62.6
	L.c.	5	21.1	3.1	17.4-25.1
Condie	A.l.	3	63.0	8.6	41.7-84.3
	D.o.	16	66.2	7.7	62.1-70.3
	P.m.	11	47.3	12.7	38.8-55.8
	L.c.	5	20.0	3.9	15.2-24.7
Fish Springs	A.l.	2	63.5	0.0	63.5-63.5
	P.m.	28	48.6	9.5	44.9-52.3
	L.c.	7	21.1	3.0	18.3-23.9
Gold Hill	D.o.	8	67.5	18.6	52.0-83.1
	P.m.	14	31.7	8.4	26.9-36.6
	L.c.	6	20.4	2.5	17.8-23.0
Government Creek	A.l	3	67.1	28.1	-2.8-136.9
	D.o.	25	60.6	23.7	50.9-70.4
	P.m.	4	48.3	8.6	34.6-61.9
	L.c.	6	20.4	3.7	16.5-24.3
Granite Mtn.	A.l.	3	77.0	4.1	66.9-87.0
	D.o.	2	70.2	3.8	35.9-104.5
	P.m.	18	48.5	10.2	43.4-53.5
	L.c.	6	19.0	4.1	14.7-23.4
Iosepa	P.m.	6	48.6	7.2	41.1-56.2
	L.c.	5	19.7	3.1	15.9-23.5
Wendover	A.l.	4	69.9	32.7	17.9-121.8
	D.o.	11	59.9	20.5	46.1-73.7
	L.c.	7	21.3	5.4	16.3-26.2

*Activities for *Lepus californicus* are reported as μM CO_2 evolved per 100 μl RBC's per 30 minutes. Activities for all other species are reported as μM CO_2 evolved per 50 μl RBC's per 30 minutes.

**Species: A.l. - *Ammospermophilus leucurus*, D.o. - *Dipodomys ordii*
P.m. - *Peromyscus maniculatus*, L.c. - *Lepus californicus*

Table 4. ERYTHROCYTE ACETYLCHOLINESTERASE ACTIVITY MEAN VALUES* IN SHEEP AND CATTLE IN BONNEVILLE BASIN OF WEST CENTRAL UTAH FALL 1970

Herd Number	Species	Summer Range	Winter Range	Mean	Sample Size	Standard Deviation	95% Confidence Interval
1	Sheep	Bear Lake	E. Dugway Mtns.	100.5	28	13.0	95.4.105.5
2	Sheep	Bear Lake	Big Davis Mtn.	98.4	30	14.0	93.2-103.6
4	Sheep	Bear Lake	White Rock	97.6	30	14.9	92.0-103.2
6	Sheep	Strawberry	E. Topaz Mtn.	77.2	24	12.5	71.9-82.5
28	Cattle	Callao	Callao	218.4	50	41.9	206.4-230.3
29	Cattle	Grouse Creek	Grouse Creek	253.5	44	55.2	236.7-270.3
30	Cattle	Vernon	Vernon	256.7	30	44.1	240.3-273.2

*Activity mean values for sheep are reported as μM CO_2 evolved per 100 μl RBC's per 15 minutes. Activity mean values for cattle are reported as μM CO_2 evolved per 50 μl RBC's per 15 minutes.

Table 5. ERYTHROCYTE ACETYLCHOLINESTERASE ACTIVITY MEAN VALUES* IN SHEEP AND CATTLE IN BONNEVILLE BASIN OF WEST CENTRAL UTAH SPRING 1971

Herd Number	Species	Summer Range	Winter Range	Mean	Sample Size	Standard Deviation	95% Confidence Interval
1	Sheep	Bear Lake	E. Dugway Mtns.	115.6	30	14.3	110.2-120.9
2	Sheep	Bear Lake	Big Davis Mtn.	105.2	30	18.3	98.4-112.0
4	Sheep	Bear Lake	White Rock	118.9	30	19.3	111.8-126.1
6	Sheep	Strawberry	E. Topaz Mtn.	100.9	30	13.0	96.0-105.8
28	Cattle	Callao	Callao	272.3	29	37.9	257.9-286.8
29	Cattle	Grouse Creek	Grouse Creek	286.9	30	53.9	266.7-306.9
30	Cattle	Vernon	Vernon	276.3	23	45.8	256.6-296.1

*Activity mean values for sheep are reported as μM CO_2 evolved per 100 μl RBC's per 15 minutes. Activity mean values for cattle are reported as μM CO_2 evolved per 50 μl RBC's per 15 minutes.

Table 6. ERYTHROCYTE ACETYLCHOLINESTERASE ACTIVITY MEAN VALUES* IN SHEEP AND CATTLE IN BONNEVILLE BASIN OF WEST CENTRAL UTAH FALL 1971

Herd Number	Species	Summer Range	Winter Range	Mean	Sample Size	Standard Deviation	95% Confidence Interval
1	Sheep	Bear Lake	E. Dugway Mtns.	94.6	30	12.2	89.9-99.1
2	Sheep	Bear Lake	Big Davis Mtn.	99.9	30	13.9	94.7-105.1
4	Sheep	Bear Lake	White Rock	97.6	30	13.9	92.4-102.7
6	Sheep	Strawberry	E. Topaz Mtn.	89.4	30	15.2	83.7-95.1
23	Sheep	Lost Creek	Gold Hill	87.1	30	13.4	82.0-92.1
33	Sheep	Coalville	W. Dugway Mtns.	88.1	30	14.0	85.9-96.4
29	Cattle	Grouse Creek	Grouse Creek	285.4	30	42.2	269.7-301.2
30	Cattle	Vernon	Vernon	285.0	23	41.7	266.9-303.0

*Activity mean values for sheep are reported as $\mu M\ CO_2$ evolved per 100 μl RBC's per 15 minutes. Activity mean values for cattle are reported as $\mu M\ CO_2$ evolved per 50 μl RBC's per 15 minutes.

Table 7. ERYTHROCYTE ACETYLCHOLINESTERASE ACTIVITY MEAN VALUES* IN SHEEP AND CATTLE IN BONNEVILLE BASIN OF WEST CENTRAL UTAH SPRING 1972

Herd Number	Species	Summer Range	Winter Range	Mean	Sample Size	Standard Deviation	95% Confidence Interval
1	Sheep	Bear Lake	E. Dugway Mnts.	96.2	30	12.3	91.6-100.8
2	Sheep	Bear Lake	Big Davis Mtn.	97.1	30	13.8	91.8-102.3
4	Sheep	Bear Lake	White Rock	94.5	30	15.4	88.8-100.3
6	Sheep	Strawberry	E. Topaz Mtn.	103.1	30	14.7	97.6-108.5
23	Sheep	Lost Creek	Gold Hill	87.6	30	12.8	82.8-92.3
33	Sheep	Coalville	W. Dugway Mtns.	94.8	29	14.2	89.4-100.2
28	Cattle	Callao	Callao	247.1	30	57.7	233.0-261.2
29	Cattle	Grouse Creek	Grouse Creek	227.9	30	36.5	214.3-241.5
30	Cattle	Vernon	Vernon	233.7	30	36.2	220.2-247.2

*Activity mean values for sheep are reported as $\mu M\ CO_2$ evolved per 100 μl RBC's per 15 minutes. Activity mean values for cattle are reported as $\mu M\ CO_2$ evolved per 50 μl RBC's per 15 minutes.

fall within a narrow range. Somewhat more variable results were obtained from *Dipodomys ordii* while *Ammosphermophilus leucurus* was often not collected in large enough numbers to be useful for statistical analysis.

CONTROL STUDIES IN SUPPORT OF FIELD PROGRAM

Results obtained from range sheep and Hereford steers following acute and chronic oral doses of the military agent VX confirm that the red cell activity recovery rate can be predicted and suggest that, by collecting a series of blood samples over a period of time under field conditions, a post-exposure diagnosis of both the degree of contamination and the time of exposure could be ascertained.

Acetylcholinesterase Determinations Using Hereford Steers

Hereford steers can tolerate the ingestion of substantial quantities of nerve agent for an extended period of time. No significant signs of illness were observed in animals receiving daily VX doses of 0.7 micrograms per kilogram body weight for 75 consecutive days, although the erythrocyte AChE activity was markedly depressed. Following 1.0 and 2.2 micrograms per kilogram per day, the only gross clinical sign was some salivation, dose-related in its degree, which resulted in frequent licking and resultant rash on the noses of the animals. Following daily treatment with 4.4 micrograms per kilogram, signs of impaired health were noted after the fifth day. At all except the lowest dosage level used (0.1 micrograms per kilogram per day), the degree of AChE inhibition was related to the total dose rather than the daily dose, i.e., a given total dose had the same effect whether administered at 0.2 or 2.2 micrograms per kilogram. However, at the low dosage, 0.1 microgram per kilogram, depression of enzymic activity was only about half as effective for a given total dose. Packed cell volumes and reticulocyte counts remained unaffected relative to control values. The blood cell observations indicate that neither the amount of circulating erythrocytes nor their lifespan or turnover time was affected by the treatment.

Data plotted in Figure 3 illustrate that erythrocyte acetylcholinesterase responds rapidly and sensitively to low intakes of VX. As seen in Figure 4, erythrocyte acetylcholinesterase activity was depressed to 62 percent when 0.1 µg/kg/day was administered for 56 days (Curve A), and to approximately 19 percent when 0.2 (Curve B) and 0.3 µg/kg/day (Curve C) were fed. At the lower dosage, there is clear evidence that the activity had reached a plateau within about 40 days, indicating the existence of a steady state in which the influx of fully active cells balanced the inhibitory action of the nerve agent. In Figures 4 (Curve D), 5, and 6, presenting enzymic activity as affected by the feeding of 0.7, 1.0 and 2.2 µg/kg/day, respectively, the AChE levels seem to have attained steady states seen most clearly in Figure 3, however, this may be more apparent than real. The activity could have been depressed to as low a value as possible, but a residuum was consistently measurable. Following a dose of 4.4 µg/kg/day, the acetylcholinesterase was rapidly depressed to nil and

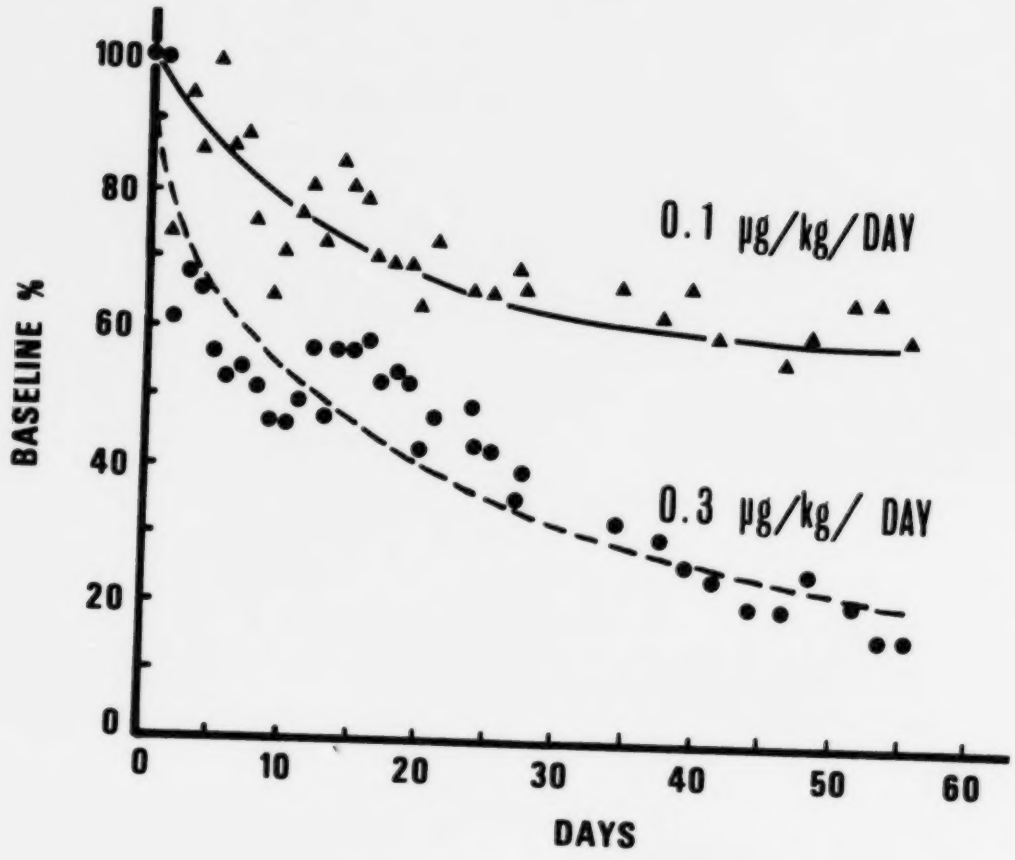


FIGURE 3. Mean dose response curve showing the relationship between oral VX exposures of 0.1 and 0.3 µg/kg/day for 56 days and the level of erythrocyte acetylcholinesterase activity. Four Hereford steers were used in each treatment group.

on several days between treatment day 45 and 75 no activity was detected. This was the only treatment (4.4 µg/kg/day for 75 consecutive days) to cause death in one of the test animals. The lack of any detectible activity was not a consistent finding during this heavy chronic exposure and this failure of enzymic activity to remain below the detection limit may be attributed to the continual turnover of erythrocytes in which new active cells replace those whose acetylcholinesterase activity was inhibited.

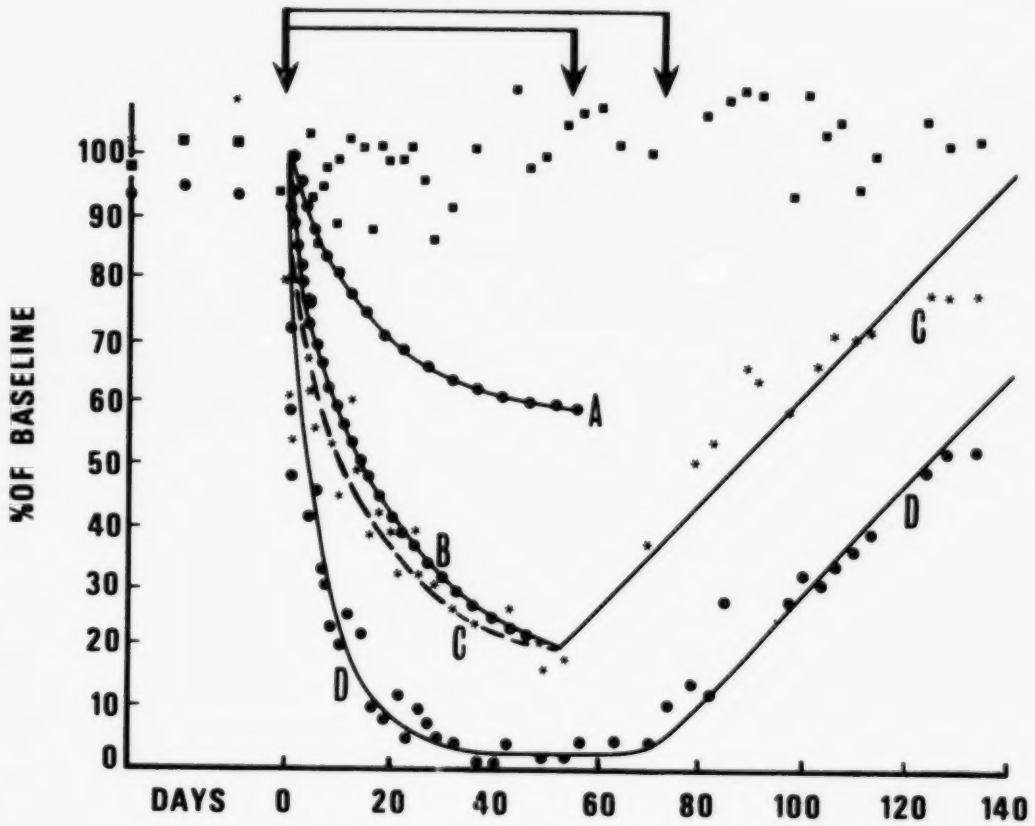


FIGURE 4. Mean dose response curve, depression and recovery phases in Hereford steers showing the relationship between oral VX exposures of 0.1, 0.2, and 0.3 µg/kg/day for 56 days plus an exposure of 0.7 µg/kg/day for 75 days and the level of erythrocyte acetylcholinesterase activity.

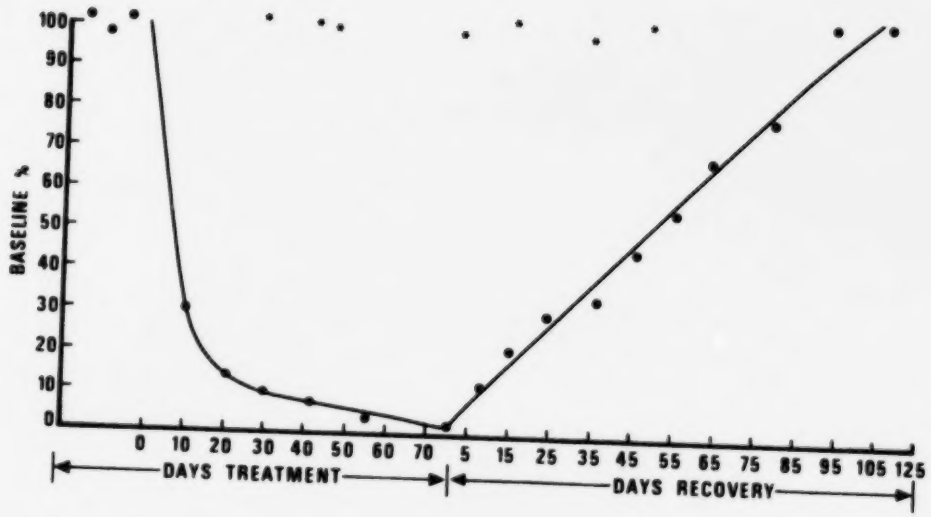


FIGURE 5. Mean dose response curve, depression and recovery phases, in three Hereford steers showing the relationship between oral VX doses of 1.0 µg/kg/day for 75 days and the level of erythrocyte acetylcholinesterase activity.

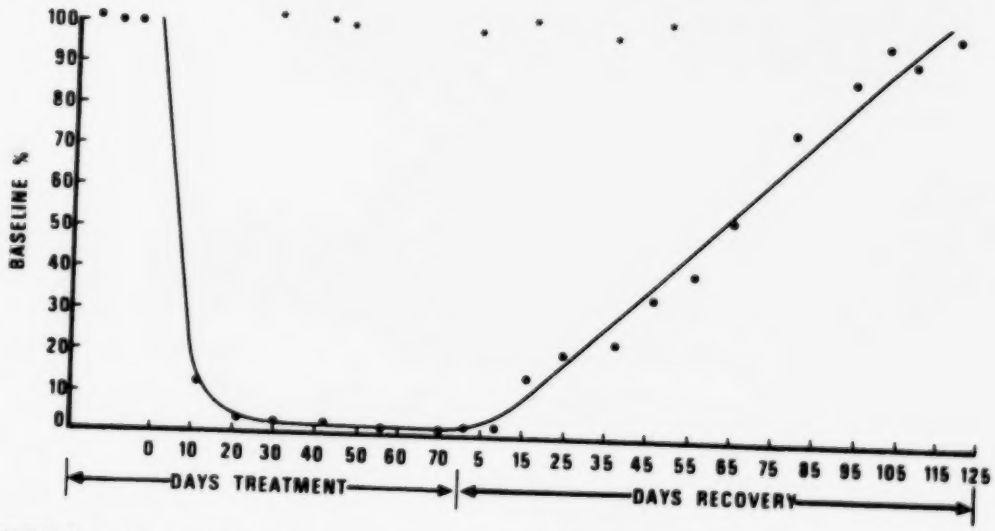


FIGURE 6. Mean dose response curve, depression and recovery phases, in three Hereford steers showing the relationship between oral VX doses of 2.2 µg/kg/day for 75 days and the level of erythrocyte acetylcholinesterase activity.

The repeated daily ingestion of VX resulted in progressive depression of AChE and, since there was insufficient time for the restoration of activity between exposures, the effect on the enzyme was virtually cumulative. Following completion of the organophosphorus treatment, the erythrocyte AChE activity returned to the original levels in an essentially linear manner, a process which covered approximately 115 days. Since erythrocyte AChE remained depressed for prolonged periods after the disappearance of symptoms, cholinesterase activity in the tissues was probably restored prior to red cell recovery. Once the restoration of red cell activity had begun the rates were independent of the amount originally ingested and the recovery of AChE activity reflected the rate of erythrocyte replacement.

Toxicity of VX in Hereford Steers and Sheep

It was concluded from a brief series of experiments involving the feeding of VX that 1.2 mg of VX per 46 kg of body weight constituted an approximate acute LD_{50} in younger steers (average weight = 251 kg). The small number of larger steers (approximate weight = 636 kg) available for this investigation did not permit estimation of an LD_{50}. There was indication that the LD_{50} value of VX may be somewhat lower for smaller steers.

The oral LD_{50} of VX in sheep was found to be virtually identical to that in cattle (Table 8), but it did not depend on body weight. VX applied directly to the skin was also quite toxic, the acute LD_{50} being approximately equal to the acute oral LD_{50}, but the time to effect (or death) was much prolonged. Interestingly, sheep can tolerate large doses of VX on wool without noticeable effects. It was clear that the wool served as an excellent protective covering. The toxicity of VX was greatest by the intravenous route (to simulate inhalation exposure) in terms of time to effect (TE), time to death (TD), and the dose required to produce death in 50% of the sheep (LD_{50}).

The lower half of Table 8 summarizes data based on the feeding of VX until death. The indicated doses were given in three divided, equal amounts at 0800, 1200, and 1600 hours daily. Although this was an artificial condition, the animals being incapable of ingesting VX following collapse on the range, it was noted that TE_{50} and TD_{50} were not grossly different at any dosage level and tended to converge as the dosage increased. TE_{50} was chosen conservatively, the time to effect being that when animals first showed signs of weakness (stumbling and loss of coordination) rather than permanent collapse. The blood cholinesterase activities of all animals declined drastically. Cholinesterase activity in animals receiving 150 µg of agent per day were below the detection limit for several days before any evidence of weakness was noted.

Additional groups of range sheep were fed VX impregnated pellets in single oral doses, multiple oral doses until collapse, and multiple oral doses until death. During these studies the sheep were maintained

Table 8. TOXICITY OF VX IN SHEEP

ACUTE

Route	TE[a]	TD[b]	LD_{50} (µg/50 kg)
I.V.	1-20 min	5-90 min	190
Skin (nose & feet)	10-60 min	23-120 min	1650
Wool	-	-	22,500
Oral	15-300 min	35 min-72 hrs	1,500

CHRONIC*

Oral (µg/day)	TE_{50}[a]	ED_{50}[c] (µg/50 kg)	TD_{50}[b]	LD_{50} (µg/50 kg)
150[d]	288 hrs	1,800	426 hrs	2,663
300[e]	69 hrs	856	88 hrs	1,100
900[f]	26 hrs	986	31 hrs	1,155

*Total dose administered in three equal doses per day (in gelatin capsules with feed).

a. TE = Time to effect (loss of coordination, ability to run or stand)
b. TD = Time to death
c. ED = Effective dose
d. Mean weight - 60 kg
e. Mean weight - 38 kg
f. Mean weight - 54 kg

at different locations on, and several miles away from, the proving ground to ascertain potential complicating environmental effects. These environmental differences primarily concerned variations in the type of pasture available to the animals. However, the different pasture conditions produced no noticeable change in the susceptibility of sheep to the VX pellets.

Cattle as Indicator Animals

For purposes of biological monitoring, cattle would seem to be excellent indicator animals since chronic daily doses of about 25 micrograms depressed enzymic activity significantly. Based on an average intake of 20 kg of feed per day*, cattle surveys will readily detect the persistent presence of 1 part of VX (or toxic substances equivalent to VX) in 1 X 10^9 parts of feed (1 part per billion). At a daily intake of 225 micrograms, less than 1 microgram per kg of body weight or 1 part per 10^8 (100 million) of feed, the effect on red cell AChE is rapid and dramatic. Only short-term exposure is needed for a telling effect. Cattle also tend to consume preferentially the upper parts of browse which are more likely to be contaminated with organophosphorus pesticides from spray operations than parts near the ground.

Acetylcholinesterase Determinations Using Range Sheep

Rambouillet-Columbia range sheep were also used as test animals to monitor the depression and subsequent recovery of erythrocyte AChe activity following acute ingestion of VX. As was the case with Hereford steers, an essentially linear return to baseline AChE activity was observed following cessation of organophosphorus exposure. Synthesis of new erythrocytes and the concomitant increase in activity was responsible for the recovery to baseline levels. Therefore, the recovery rate is largely independent of the original degree of depression caused by the cholinesterase inhibitor and the length of time required for the recovery will be approximately the same whether the original degree of depression was 20 per cent or 90 per cent.

Results from Studies Using Laboratory Animals

It has been shown that guinea pigs are suitable bioassay tools. They reflect repeated oral intakes of about 1 microgram of VX per kg per day by significant red cell AChE depressions. While this is far

*The Merck Veterinary Manual (1961) estimates the daily consumption of feed by 600-pound beef steers to be 16 pounds (7.7 kg) of 90% dry weight material. The present calculation is based on the assumption that dry matter constitutes 33% of the weight of forage plants.

lower than the sensitivity of chemical assay, VX, or the equivalent in toxic substances derived therefrom, may be distributed throughout the relatively bulky amount of food consumed by the animals. As with domestic animals and wildlife species, the specific identity of the organophosphorus substances need not be known for this purpose, so that guinea pigs may serve as a screening mechanism. It is expected that a drawback in this method will be the dietary fastidiousness of the animals.

The domestic rabbit appears to be a relatively poor candidate for bioassay of VX and its toxic products at low concentrations. Quantities of VX that had a decided effect on AChE in guinea pigs had little effect on the enzymic activity of rabbit erythrocytes. The conclusion that the domestic rabbit is a poor bioassay tool is reinforced by the fact that AChE in rabbit erythrocytes is intrinsically low.

Studies on Wildlife Species (Small Mammals)

Controlled laboratory studies were also conducted using deer mice and jackrabbits and included a very limited effort with the wood rat and Ord kangaroo rat. Data resulting from these studies to date have not revealed the clear sequence of AChE depression and subsequent recovery following oral VX challenges. The red cell AChE activity levels were significantly depressed following VX administration but individual activity values were very erratic. It should be stressed that the techniques required for the controlled studies on wildlife species are somewhat more difficult than the relatively simple feeding and sample collecting steps performed on domestic and laboratory animals. Frequent collection of blood from a small mammal such as the deer mouse can stimulate erythropoiesis and complicate the red cell AChE picture. Furthermore, wild mammals such as the jackrabbit can be difficult to handle during the organophosphorus feeding trials. The only conclusions that can be reached at this time are that red cell activity is sensitive to organophosphorus exposure and these wild animals appear to be suitable field indicators for the presence of such anticholinesterase compounds. Dose-response curves still need clarification, however.

Studies on Wildlife Species (Fish)

The manometric technique has also been employed to study the baseline levels of brain acetylcholinesterase activity in rainbow trout from five hatcheries operated by the Utah Department of Natural Resources, Division of Fish and Game. Ninety-five samples of trout ranging in length from 61 to 368 mm and in weight from 3 to 482 g have been studied as a function of wet brain weight as well as total brain protein.

Rainbow trout brain acetylcholinesterase activities range from 0.5 to 1.4 micromoles of acetylcholine hydrolyzed per mg wet brain tissue per hour, and from 6.4 to 16.0 micromoles of acetylcholine hydrolyzed per mg total brain protein per hour, depending upon the

size of the trout. The brain acetylcholinesterase activity has been found to decrease as the wet brain weight, total fish length and fish weight increase.

Curves for the limits of rainbow trout brain acetylcholinesterase activity have been determined by calculating the arithmetic means (confidence coefficient = 0.95) for activities and wet brain weight, total length, and weight classes of trout.

With these background data, enzyme inhibition due to unusual occurrences of organophosphorus compounds in the aquatic environment can be detected.

Complementary Toxicological Effects of Plant Poisons and Chemical Agents on Mammals

Poisonous plants are common to the grazing ranges surrounding DPG and are known to be consumed by livestock, occasionally in lethal quantities. It is therefore essential to establish whether the toxicity of these poisonous plants and that of VX are additive or even synergistic. In this way it may be established whether organophosphorus pesticides or nerve agents represent an especially acute hazard to animals whose feed contains toxic plant substances.

During a series of studies designed to elucidate this problem (1970-1972), combinations of organophosphorus agent and plant substance(s) were administered, consecutively or simultaneously, to determine interactions of the poisonous substances in terms of gross physiological effects. Data were collected to permit estimation of seasonal risk factors by location at the Dugway perimeter.

1. Oxalate-containing plants are common in West Central Utah. Halogeton (*Halogeton glomeratus*) is a frequent problem to ranchers and greasewood (*Sarcobatus vermiculatus*) to a more limited extent. When appreciable quantities of these plants are consumed, the soluble oxalate may either unite with the calcium in blood and upset the mineral balance in the interstitial fluid producing hypocalcemia, be degraded by rumen microorganisms, or combine with calcium in the rumen to form insoluble calcium oxalate. The reduction in serum calcium is mainly caused by the deposition of calcium oxalate in the soft tissues. Death usually results from a combination of factors including tissue damage, hypocalcemia, interference with energy metabolism, asphyxiation, and heart failure because of changes in membrane potential. In summary, the literature yielded little information that would suggest any direct pharmacologic interaction between oxalate and VX. However, changes in free (ionic) calcium are known to affect neuromuscular irritability and the release of acetylcholine from presynaptic vesicles, in this way establishing a tenuous link to the primary action of VX. Furthermore, there remained

the strong possibility that a debilitated animal is rendered more susceptible to low quantities of VX (and other anticholinesterase compounds) in an essentially nonspecific way.

Over 100 Rambouillet-Columbia sheep were studied at DPG during 1970 and 1971 to investigate potential synergistic responses following oral doses of oxalate and VX. The study was supported by laboratory analyses to determine (1) red cell acetylcholinesterase (AChE) levels during and after treatment and (2) serum calcium, phosphate and creatinine values. Numerous necropsies were performed and a rather extensive series of histological examinations were made on selective tissues.

The most consistent pathological finding following oxalate ingestion (with or without a VX combination) was hemorrhage and edema of the rumen and reticulum with destruction of the rumenal arteries by oxalate crystals. Hemorrhagic alterations in the reticulum were neither pronounced nor consistently present. The crystals were distributed throughout the duct system and cell degeneration was occasionally noted adjacent to the areas of crystalization. Blood-tinged froth was commonly present in the nasal passageways and the lungs were usually congested. Hypocalcemia, hyperphosphatemia and slightly elevated creatinine values, indicating some retention of nitrogenous substances, were also noted in sheep that had ingested oxalate. Red cell AChE levels were, as expected, not effected by the oxalate treatments.

Animals receiving various VX challenges showed the classic responses to organophosphorus intoxication. Red cell AChE recovery following VX doses was not effected by a simultaneous administration of oxalate during the treatment period. Total AChE recovery took approximately 120 days. Post-mortem examinations conducted on sheep treated with VX alone revealed no signs of tissue damage. In one case lung congestion was pronounced but the animal in question was recumbent for 12 hours prior to death.

When oxalate and VX were administered simultaneously there was some evidence of synergism in the mortality ratio. However, the apparent potentiating effect was not catastrophic. From the point of view of chemical safety, the presence of oxalate containing plants in grazing areas does not create unforeseen chemical safety hazards to range sheep since only large intakes of oxalate, VX or combinations of these are likely to eventuate in death. There is however, a greater risk of misdiagnosis of symptoms. Quantities of VX far below the fatal level, with or without oxalate, can supress erythrocyte AChE activities to nil. Therefore, enzymatic activity is an inadequate criterion in establishing the cause of death, and major reliance must be placed on other signs and symptoms for a definitive diagnosis.

2. Western false hellebore *(Veratrum californicum)*, a large coarse, erect herbaceous plant with broad leaves, is found in mountain meadows and valleys near DPG where it often invades and dominates moist slopes. In cases of serious depletion it is frequently one of the last perennial species in the meadow association to disappear. The toxic substance is composed of a rather large group of chemically related alkaloids, and the resulting toxicity following the ingestion of plant material is influenced by several variables, e.g., stage of growth. Following veratrum ingestion the animal demonstrates symptoms that are similar to those resulting from VX intoxication and include general weakness, excessive salivation with frothing, and irregular gait, coma and extensor rigidity. The veratrum alkaloids did not depress red cell AChE activity. Veratridine, an ester alkaloid, germine, an alkamine, and veratrine, a mixture of alkaloids and possibly the most representative test material, were injected at various dose levels (without and with a VX combination) into Swiss-Webster mice. The experiments on mice were pilot studies only and complete dose-response curves were not established for each veratrum alkaloid, but possible synergistic responses were noted in the trials when VX was administered concurrently.

A synergistic response, as revealed by total deaths, was obtained in sheep that received both VX and veratrine. However, when the number of severely affected animals was included for comparison, the results indicated an addition of of individual toxicities rather than a potentiation. Thus, while an increased hazard apparently exists for sheep that are exposed to both VX and veratrum plants, the study is not entirely realistic because relatively large doses of agent VX were used. Corresponding levels of contamination would be considered dangerous in themselves and, therefore, this study did not suggest a need for revised safety standards. A major problem in the field would be an incorrect diagnosis since neither veratrine nor VX cause pathological lesions and both toxins produce nearly identical gross symptoms. An erythrocyte AChE analysis will determine if organophosphorus agents are involved but will not provide information on complications due to veratrum plants.

3. Death camas *(Zigadenus spp.)*, a poisonous Utah range plant, is often found near Dugway in areas where, judging by the tracks and accumulation of feces, sheep have obviously been grazing. Morphological characteristics of the plant include long narrow leaves, an unbranched single stem, a terminal raceme or panicle of greenish white, yellow or pink flowers, and a scaly onion-like underground bulb (but without onion odor). The ester alka-

loids of death camas possess pharmacological activity resembling the veratrum alkaloid veratridine while the alkamine germine is found in both false hellebore and death camas. Since *Zigadenus* and *Veratrum* species are included in the same subfamily (*Melanthiaceae*) of liliaceous plants, it is not surprising that their respective alkaloids are of a similar chemical composition. The majority of sheep losses attributed to death camas occur in early spring because the plant furnishes green, succulent feed in advance of many range species, and because the young plant stages are the most toxic to livestock.

Initially, a series of field trips was conducted to establish the relative frequency of death camas in the Dugway area. Since extraction procedures do not provide a high yield of toxin, plant material was homogenized in a Waring blender and fed to sheep with stomach tubes. Feeding poisonous plants with a stomach tube was apparently successful and the study revealed some indication of potentiation when oral doses of death camas and agent VX were given concurrently. However, the probable result was that the death camas challenge so debilitated the animal that the additional VX insult caused death without being the result of classical synergism.

Evaluation of Complementary Effects Between Military Chemical Agents and Organophosphorus Pesticides

Consideration has been given to the possibility that certain combinations of military chemical agents and organophosphorus agricultural poisons might have additive toxic effects since they often have a similar mechanism of action. It should be noted that the brief experiments reported here were not conducted in great detail since supporting information only was required for the DPG monitoring program. However, in considering the application of agents attention was directed toward, among other things, the time of occurrence of maximal depression of red cell activity for each compound and the individual rates of absorption and detoxification.

Cattle and sheep were treated with agricultural pesticides following the method and dosage recommended by the manufacturer. The response to the pesticide and military agent given alone was compared to the response when treatment consists of a combination (military agent and pesticide) dose. Modifications of this procedure included the response to the combination challenge when excessively high doses of pesticide were given (a condition that might occur, if the ranchers do not follow the manufacturer's advice).

An indication of potentiation was observed when Bayer 21/199 pesticide and agent VX were simultaneously administered to mature sheep. Future experiments might apply the pesticide approximately three days

prior to the VX challenge since the oral VX dose takes effect more rapidly than the percutaneous pesticide treatment. While by definition this experiment revealed a suggestion of synergism, this conclusion should be viewed with caution because of the small number of sheep per group and the absence of a complete Bayer dose-response curve.

Selected levels of VX and/or malathion were also administered to Rambouillet-Columbia sheep for the purpose of monitoring the red cell acetylcholinesterase response and to investigate the possibility of synergism resulting from the two organophosphorus substances. These two compounds did not produce a synergistic action. However, they did produce an additive effect. Sheep that survived the combination malathion-VX treatment had typical erythrocyte AChE recovery curves.

REFERENCES CITED

1. Anderson, P. H., A. F. Machin, and C. N. Herbert, "Blood Cholinesterase Activity as an Index on Acute Toxicity of Organophosphorus Pesticides in Sheep and Cattle," Res. Vet. Sci., Vol. 10, pp. 29-33, 1969.

2. Augustinsson, K., "Assay Methods for Cholinesterase," Methods of Biochemical Analysis, Vol. 5, pp. 1-63, 1954.

3. Gaballah, S., "A Direct Radioisotopic Microassay for Cholinesterase," Proc. Soc. Exp. Biol. Med., Vol. 129, pp. 376-380, 1968.

4. Holmstedt, B., Structure-Activity Relationships of the Organophosphorus Anticholinesterase Agents, p. 428-485, In Cholinesterases and Anticholinesterase Agents, G. B. Koelle, Ed., Springer-Verlag, Berline-Gottingen-Heidelberg, 1220 pp., 1963.

5. Merck Veterinary Manual, Second Edition, Merck and Co., Inc., Rahway, NJ, 1630 pp., Beef Cattle Nutrition, p. 694-706, 1961.

6. Reed, D. J., K. Goto, and C. H. Wang, "A Direct Radioisotopic Assay for Acetylcholinesterase," Analytical Biochemistry, Vol. 16, pp. 59-64, 1966.

7. Umbreit, W. W., R. H. Burris, and J. F. Stauffer, Manometric Techniques and Tissue Metabolism, Burgess Publishing Co., Minneapolis, MN, 227 pp., 1949.

8. Witter, R. F., "Measurement of Blood Cholinesterase," Archives of Environmental Health, Vol. 6, pp. 537-562, 1963.

APPENDIX I

DESCRIPTIVE OUTLINE OF THE WARBURG MANOMETRIC TECHNIQUE USED FOR THE ASSAY OF RED BLOOD CELL ACETYLCHOLINESTERASE ACTIVITY

1. Preparation and Storage of Erythrocytes Prior to Assay for Acetylcholinesterese Activity

Blood from large mammals is drawn in 10-ml heparinized Vacutainer tubes and mixed by inverting each tube several times to prevent clotting. Small mammal blood is collected in 3-ml heparinized Vacutainer tubes. The blood is stored at 4° C until processing. When processed, the tubes of whole blood are centrifuged in a refrigerated centrifuge (4° C) at 3,000 rpm for for 12 minutes. The plasma, the white blood cells and any clots are removed from the surface of the packed red blood cells and discarded.

Two volumes of 0.9% sodium chloride solution are added to one volume of packed red blood cells in tubes. The cells and saline are gently mixed by inverting the tubes several times and centrifuged at 3,000 rpm for 12 minutes. Supernatant fluid should be drawn off slowly and carefully in order to remove all of the saline solution but as little of the red blood cells as possible. This washing procedure removes serum cholinesterase activity and other potentially interfering substances.

Red blood cells from cattle and sheep should be washed to remove all clotting substances even though the plasma has little or no detectable cholinesterase activity. Whole erythrocytes of small mammals should be stored at 4° C until time of assay.

For preparation of sheep erythrocytes 2.1 ml of 0.1% saponin solution is pipetted into clean plastic vials. One ml of the erythrocyte suspension is added to the 2.1 ml of saponin solution. Saponin solution lyses the red blood cells and liberates cell bound enzyme. Rinse the pipette several times by repeatedly aspirating and expelling the saponin solution-erythrocyte mixture. Allow the lysed cells to stand 10 to 15 minutes at room temperature and freeze the tubes at -27° C until time of assay. The remainder of the non-lysed cells may be stored along with the lysed cells for future reference.

Sample preparation of cattle erythrocytes consists of pipetting 2.6 ml of 0.05% saponin solution into clean plastic vials. Draw up 0.5 ml of the erythrocyte suspension and add this to the saponin solution. Rinsing the pipette and storage procedured are the same as for the sheep erythrocytes.

2. Preparation and Storage of Enzyme-Inhibited Erythrocytic Controls

Add 15 ml of distilled water to a clean 50-ml Erlenmeyer flask. Add one 15-mg tablet of prostigmin (a cholinergic drug used in the form of the bromide, Roche Laboratories, Nutley, NY) to the flask. Allow five minutes for the tablet to dissolve, mix by gently swirling the flask, and filter the solution. Measure 1.05 ml of 0.1% saponin solution into each plastic vial. Add 1.05 ml of the filtered prostigmin solution into each vial. Draw up 1.0 ml of the appropriate erythrocyte suspension (fresh whole washed rabbit, cattle or sheep erythrocytes) and add this to the solution in each of the vials. Excess blood must be wiped from the tip and outer surfaces of the pipette, otherwise, the sample size will vary considerably. As before, rinse the pipette and store the vials of enzyme-inhibited erythrocytes at -27° C until time of assay.

These preparations provide 100 microliters (µl) of enzyme-inhibited lysate per 0.3 ml, the amount required as a control in the assay of sheep and rabbit erythrocytic specimens. For specimens from cattle and rodents only 0.15 ml of the lysate is required to equal 50 µl quantity specified. Because of the small quantities of blood obtained from rodents, lysates prepared from rabbit erythrocytes are employed as controls for rodent specimens.

3. Preparation of Reagents for Manometric Assay for Erythrocyte Acetylcholinesterase Activity

The reagents required for manometric assay of erythrocyte acetylcholinesterase activity are (1) modified Krebs-Ringer (KR) solution prepared fresh daily and (2) acetylcholine iodide prepared fresh daily in KR solution. The Krebs-Ringer solution is saturated for at least 15 minutes with a mixture of 95% N_2 + 5% CO_2. (Attach a fritted glass stick with plastic tubing to the gas regulator on the gas cylinder, and release a small but steady stream of the gas mixture.) The concentration of acetylcholine iodide depends on the particular species from which specimens are taken. For rodent specimens a 0.011 M solution is prepared. Add 150.2 mg acetylcholine iodide to a 50 ml volumetric flask, and fill to the mark with gassed Krebs-Ringer solution. For cattle, sheep and jackrabbit specimens a 0.01 M solution is prepared. Add 136.6 mg acetylcholine iodide to a 50 ml volumetric flask and fill to the mark with gassed Krebs solution.

4. Preparation of Samples for Assay

a. Lysate Samples from Cattle and Sheep Erythrocytes

Allow the erythrocytic lysates to thaw at room temperature for at least one hour. Once thawed, mix the individual samples gently. With a 1-ml serologic pipette add 0.3 ml of each sample to be assayed into the sidearm of each individual Warburg flask; accurate measurement is essential. Fill the pipette and wipe excess blood from the sides and tip before dispensing. Do not dispense the last 0.1 ml in the pipette. Add 2.2 ml of 0.01 M acetylcholine iodide solution to the main compartment of each Warburg flask. Prepare a negative control in one flask to serve as a thermobarometer control as follows: for sheep erythrocytic lysates, pipette 0.3 ml enzyme-inhibited lysate into the sidearm. Into the main compartment pipette 2.2 ml of 0.01 M acetylcholine iodide solution. For cattle erythrocytic lysates, pipette 0.15 ml enzyme-inhibited lysate plus 0.15 Krebs-Ringer solution into the sidearm. Pipette 2.2 ml of 0.01 M acetylcholine iodide solution into the main compartment.

b. Erythrocytic Samples from Jackrabbits or Domestic Rabbits

Using a 1.0 ml serologic pipette dispense 0.2 ml of a 0.1% saponin solution into the sidearm of a Warburg flask. With a 100-µl micripipette draw up 100 µl of whole erythrocytes for each sample and add this to the 0.2 ml saponin solution in the sidearm of the appropriate flask. Measure the amount accurately and wipe excess sample from the sides and tip of the pipette. Rinse the micropipette carefully by repeatedly aspirating and expelling the saponin solution-erythrocyte mixture. Avoid creating bubbles. Let the micropipette remain standing in the sidearm for five minutes to allow all of the solution to drain. Remove the micropipette and expel any remaining solution into the sidearm. Shake the flask carefully to mix the solution in the sidearm, but avoid contaminating the main compartment of the flask with the blood mixture. If any erythrocytic mixture does contaminate the main compartment, discard the flask and begin again. Add 2.2 ml of 0.01 M acetylcholine iodide solution to the main compartment of each flask. Prepare a blank or negative control in one of the flasks to serve as a thermobarometer control. Pipette 0.3 ml enzyme-inhibited erythrocytic lysate from rabbits into the sidearm. Pipette 2.2 ml of 0.01 M acetylcholine iodide solution into the main compartment.

c. Erythrocytic Samples from Rodents

Using a 1.0-ml serologic pipette dispense 0.2 ml of a 0.1% saponin solution into the sidearm of a Warburg flask. With a 50-µl micropipette draw up 50 µl of whole erythrocytes and add

this to the 0.2 ml saponin solution in the sidearm of the flask. Measure the amount accurately and wipe excess sample from sides and tip of the pipette. Rinse the micropipette carefully by repeatedly aspirating and expelling the saponin solution-erythrocyte mixture; avoid creating bubbles. Let the micropipette remain standing in the sidearm for five minutes to allow all of the solution to drain. Remove the micropipette, and expel any remaining solution into the sidearm. With a 1.0 ml serologic pipette add 0.25 ml of the saturated Krebs-Ringer solution to the sidearm of the flask and shake each flask carefully. Pipette exactly 2 ml of 0.011 M acetylcholine iodide solution to the main compartment of the flask. Prepare a blank manometric control by placing 0.15 ml of enzyme-inhibited erythrocytic lysate from rabbits plus 0.35 ml of gas saturated Krebs-Ringer solution into the sidearm. Place 2.0 ml of 0.011 M acetylcholine iodide solution in the main compartment.

d. Assay of Samples for Acetylcholinesterase Activity

A Warburg apparatus is used to measure the evolution of CO_2. Check the level of Brodie's solution in each manometer. Add more solution if necessary to bring the level of fluid to within no more than 1/3 inch below the black index line. Check to see that all stopcocks on the manometers are open. Set each micrometer to 450. Apply lanolin to the ground glass joints of the sidearm stoppers, as well as to the ground glass joints on the manometers. Assemble the Warburg flasks to the manometers and secure each flask with a spring. Insert the venting plugs into the sidearms and check to see that the gas vents in the sidearms are open. Secure each plug with a spring. Insert the frames on the Warburg apparatus as desired. Check the level of water in the bath to insure that it reaches but does not cover the neck of the Warburg flasks. Monitor the level of fluid in each manometer as a double check that the sidearm vents are open and have remained open. Establish that the temperature of the water bath is 37° C. Connect a gassing tube to each sidearm plug and flush the vessels with the 95% N_2 + 5% CO_2 mixture. Adjust the flow of gas carefully. Use enough pressure to flood the flasks with gas, but avoid excessive pressure, which might expel or mix the separated components. Continue to flush with the gas mixture for 10 minutes. Afterwards close the gas vent in each sidearm of the vessels by rotating the plug one quarter (90°) turn, and disconnect the gassing tube. Remove each frame individually from the Warburg apparatus and tip the frames three times to mix thoroughly the substrate and blood preparations. Complete the mixing to assure that the entire contents are in the main chamber of the flasks. Replace the frames on the Warburg apparatus and start the shaker. Allow 10 minutes for temperature equilibration and the reaction to commence. Close the stopcocks on the manometer. Adjust the micrometers to bring the fluid level to the black index line on

each manometer. Read and record the micrometer settings five minutes after closing the stopcocks. Continue to adjust the micrometers as often as necessary during incubation to maintain the fluid level at the index line. Adjust the fluid level and record readings at 5-minute intervals for the duration of the assay. The duration of assay varies with the species of erythrocytes under test. Specimens from cattle and sheep are followed for 30 minutes; those from rabbits and rodents, 45 minutes. At the conclusion of the assays stop the shaker and open the stopcocks on the manometers. Reset micrometers to their initial reading (450). Remove the frames from the Warburg apparatus, remove venting plugs from the sidearms and remove the flasks from the manometers. Wipe the remaining lanolin from the ground glass joints of the manometers with tissue paper, and clean the Warburg flasks and sidearm stoppers.

5. Reporting Results

Results for cattle and sheep are recorded as µl CO_2 evolved respectively per 50 and 100 µl RBC per 15 minutes. Results for rodents and jackrabbits are recorded as µl CO_2 evolved respectively per 50 and 100 µl RBC per 30 minutes. Prepare a data test chart as shown in Appendix Table 1. Five-minute time intervals ranging from 0 to 30 minutes for livestock and 0 to 45 minutes for wildlife are placed across the top of the chart. Manometer readings are recorded in the top half of the chart. Values in the lower half are obtained by subtracting the readings of the later time interval from the earlier reading for each sample.

The system is allowed to stabilize during the first ten minutes after mixing; no readings are taken during this time period. In the case of livestock, total the readings taken for the 15, 20, and 25-minute periods. Then, in a separate step, sum those values for the 20, 25, and 30-minute periods. Compute a mean for these two subtotals and subtract the mean for the enzyme-inhibited control (IC) sample. The difference represents the net AChE activity. For specimens from wildlife species sum the values for the 15, 20, 25, 30, 35, and 40-minute intervals. Then total the values for the 20, 25, 30, 35, 40, and 45-minute intervals. As before, compute a mean for these two subtotals and subtract the mean for the enzyme-inhibited control sample. A standard control (SC) sample is prepared from sheep (or other available species) erythrocytes and is assayed as a standard with each assay of unknown. To compute the net standard control for each assay, first total the readings taken for the 15, 20, and 25-minute intervals; then sum the values for the 20, 25, and 30-minute periods. Compute a mean for these two subtotals and subtract the mean of the enzyme-inhibited control. The difference represents the net standard control for the assay. Values which were recorded as microliters (µl) are reported as micromoles (µM). The conversion factor for jackrabbits is 0.68, for rodents and sheep 1.34, and for cattle 2.68.

APPENDIX TABLE 1. ACETYLCHOLINESTERASE (AChE) DATA TEST CHART

Time intervals		00	05	10	15	20	25	30	35	40	45
					Livestock			Wildlife			
Specimen number	1	--	--	--	--	--	--	--	--	--	--
	2	--	--	--	--	--	--	--	--	--	--
	3	--	--	--	--	--	--	--	--	--	--
	4	--	--	--	--	--	--	--	--	--	--
	5	--	--	--	--	--	--	--	--	--	--
	6	--	--	--	--	--	--	--	--	--	--
Readings from manometers	7	--	--	--	--	--	--	--	--	--	--
	8	--	--	--	--	--	--	--	--	--	--
	9	--	--	--	--	--	--	--	--	--	--
	10	--	--	--	--	--	--	--	--	--	--
	11	--	--	--	--	--	--	--	--	--	--
	12	--	--	--	--	--	--	--	--	--	--
	13	--	--	--	--	--	--	--	--	--	--
	14	--	--	--	--	--	--	--	--	--	--
	15	--	--	--	--	--	--	--	--	--	--
	S.C.	--	--	--	--	--	--	--	--	--	--
	I.C.	--	--	--	--	--	--	--	--	--	--

		Omit									Avg.			Net
	1	-	-	-	-	-	-	-	-	-	--	--	--	--
	2	-	-	-	-	-	-	-	-	-	--	--	--	--
	3	-	-	-	-	-	-	-	-	-	--	--	--	--
	4	-	-	-	-	-	-	-	-	-	--	--	--	--
	5	-	-	-	-	-	-	-	-	-	--	--	--	--
	6	-	-	-	-	-	-	-	-	-	--	--	--	--
Values from readings	7	-	-	-	-	-	-	-	-	-	--	--	--	--
	8	-	-	-	-	-	-	-	-	-	--	--	--	--
	9	-	-	-	-	-	-	-	-	-	--	--	--	--
	10	-	-	-	-	-	-	-	-	-	--	--	--	--
	11	-	-	-	-	-	-	-	-	-	--	--	--	--
	12	-	-	-	-	-	-	-	-	-	--	--	--	--
	13	-	-	-	-	-	-	-	-	-	--	--	--	--
	14	-	-	-	-	-	-	-	-	-	--	--	--	--
	15	-	-	-	-	-	-	-	-	-	--	--	--	--
	S.C.	-	-	-	-	-	-	-	-	-	--	--	--	--
	I.C.	-	-	-	-	-	-	-	-	-	--	--	--	--

S.C.	--	--	--
I.C.	--	--	--

Net S.C. is ___